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Transcriptional activation of CBF β by CDK11^{p110} is necessary to promote osteosarcoma cell proliferation

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Abstract

Background: Aberrant expression of cyclin-dependent protein kinases (CDK) is a hallmark of cancer. CDK11 plays a crucial role in cancer cell growth and proliferation. However, the molecular mechanisms of CDK11 and CDK11 transcriptionally regulated genes are largely unknown.

Methods: In this study, we performed a global transcriptional analysis using gene array technology to investigate the transcriptional role of CDK11 in osteosarcoma. The promoter luciferase assay, chromatin immunoprecipitation assay, and Gel Shift assay were used to identify direct transcriptional targets of CDK11. Clinical relevance and function of core-binding factor subunit beta (CBF β) were further accessed in osteosarcoma.

Results: We identified a transcriptional role of protein-DNA interaction for CDK11^{p110}, but not CDK11^{p58}, in the regulation of CBF β expression in osteosarcoma cells. The CBF β promoter luciferase assay, chromatin immunoprecipitation assay, and Gel Shift assay confirmed that CBF β is a direct transcriptional target of CDK11. High expression of CBF β is associated with poor outcome in osteosarcoma patients. Expression of CBF β contributes to the proliferation and metastatic behavior of osteosarcoma cells.

Conclusions: These data establish CBF β as a mediator of CDK11^{p110} dependent oncogenesis and suggest that targeting the CDK11- CBF β pathway may be a promising therapeutic strategy for osteosarcoma treatment.

Keywords: CDK11, CBF β , Osteosarcoma, Transcription

Background

It has been well established that overexpression and activation of cyclin-dependent kinases (CDK) is a hallmark of human cancer [1, 2]. The Food and Drug Administration (FDA) has approved the CDK4/6 inhibitors Palbociclib, Ribociclib and Abemaciclib for treating metastatic breast cancer. These inhibitors have also demonstrated promising antitumor potentials both as a monotherapy and in combined therapy in numerous clinical trials [3–6]. CDK11 is a member of the serine/threonine protein kinase family. CDK11 plays a crucial role in cell proliferation and growth of cancers, such as breast cancer, ovarian

cancer, multiple myeloma, and sarcoma [7–12]. Knock-down of CDK11 by shRNA or siRNA inhibits cancer cell growth and induces apoptosis. In vivo administration of CDK11 siRNA was shown to reduce tumor growth in cancer xenograft mouse models [13, 14]. Importantly, nuclear CDK11 expression levels correlate with clinical prognosis in cancer patients, including breast cancer, ovarian cancer, and sarcoma [8–10, 13–15]. Downregulation of CDK11 also causes significant loss of cell viability and clonal survival in breast cancer, colon cancer, cervical cancer, multiple myeloma, and acute myeloid leukemia [9, 16–19].

There are three major CDK11 protein isoforms encoded from an identical mRNA: CDK11^{p110}, CDK11^{p58}, and CDK11^{p46}. The CDK11^{p58} isoform is generated by an internal ribosome entry site sequence (IRES) in the same mRNA encoding the CDK11^{p110} isoform. CDK11^{p46} is generated by caspase dependent cleavage of CDK11^{p110}

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and CDK11^{P58} [20, 21]. In general, these protein isoforms regulate RNA transcription and processing, mitosis, and apoptosis. CDK11^{P110} is expressed in all phases of the cell cycle, while CDK11^{P58} is expressed transiently only during the G2/M phase of mitosis. Inhibition of CDK11 specifically suppresses RNAP II-dependent transcription and can be rescued by addition of purified CDK11 [22]. However, the molecular mechanisms and signaling pathways of CDK11 regulated genes in cancer cells are largely unknown.

In the present study, we performed a global transcriptional analysis using gene array technology to investigate the transcriptional role of CDK11 in osteosarcoma. We identified CDK11^{P110}, but not CDK11^{P58}, in the transcriptional regulation of core-binding factor subunit beta (CBF β) expression in osteosarcoma cells, which is important for bone cell development and formation of the skeleton. Knockdown of CDK11 caused a strong decrease in the levels of CBF β . High CBF β expression correlated with CDK11 expression and contributed to reduced overall survival in osteosarcoma patients. Our data suggest that a direct link exists between the CDK11^{P110}-CBF β pathway and osteosarcoma cell growth and migration.

Methods

Cell lines

The human osteosarcoma cell lines U-2OS, MG63, SaOS, MNNG/HOS, and 143B were purchased from the American Type Culture Collection (Rockville, MD). The human osteosarcoma KHOS cell line was kindly provided by Dr. Efstathios Gonos (Institute of Biological Research & Biotechnology, Athens, Greece). The human osteoblast cells NHOst and HOBc were purchased from LonzaWalkersville (Walkersville, MD) and PromoCell GmbH (Heidelberg, Germany), respectively. The osteosarcoma cell lines were incubated in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum. The osteoblast cell lines were cultured in osteoblast growth medium (PromoCell) with 10% fetal bovine serum.

Synthetic CDK11 or CBF β siRNA transfection

Validation of CDK11 or CBF β knockdown phenotypes in osteosarcoma cell lines was conducted by transfection of synthetic human CDK11 (Applied Biosystems, Grand Island, NY) or CBF β siRNA (Applied Biosystems). The siRNA sequence targeting CDK11 corresponded to coding regions (5'-AGAUCUACAUCGUGAUGAAtt-3', antisense 5'-UUCAUCACGAUGUAGAUCUtg-3') of the CDK11 gene. The siRNA sequence targeting CBF β corresponded to coding regions (5'-CCGAGAGUAU GUCGACUUAATT-3', antisense 5'-UAAGUCGACA UACUCUCGGCT-3') of the CBF β gene. Transfection with siRNA was performed using Lipofectamine™

RNAiMAX (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. The non-specific siRNA oligonucleotide (Applied Biosystems) was used as a negative control. Medium was changed 24 h after transfection with RPMI 1640 supplemented with 10% fetal bovine serum. Total protein was extracted 72 h after CDK11 or CBF β siRNA transfection.

Global gene expression profiling and analysis

Total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA) and submitted to Human Genome U133 plus 2 arrays for microarray. Microarray studies were performed on KHOS and U-2OS cells that were transfected with CDK11 siRNA or non-specific siRNA. To account for and eliminate biological noise, RNA was isolated from three distinct flasks of each cell line 48 h post siRNA transfection. Total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's guidelines. The quality and size distribution of the RNA were assessed for microarray analysis. Each sample was separately hybridized on Human Genome U133 plus 2 arrays, according to the manufacturer's guidelines (Affymetrix, Santa Clara, CA). Raw Affymetrix intensity measurements were background corrected, normalized, and summarized into gene expression measurements using dChip (dchip.org; <https://www.hsph.harvard.edu/pqg/software/>). The average expression value for each gene across the arrays was used to normalize the mRNA intensities. The CEL files were transformed into intensity information using the RMA normalization of GeneSifter (http://www.genesifter.net/web/qs_analysis.html) and Qlucore 3.0 (New York, NY), and fold change was used to select differentially expressed genes [23–25]. Differentially expressed gene identifiers were uploaded into a heat map. Data are available at the database of Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>) with the access code GSE73422 [26, 27].

Plasmid constructions

GFP-CDK11^{P110} and GFP-CDK11^{P58} plasmids were kindly provided by Dr. Régis Giet. GFP-CDK11^{P110} kinase-dead or kinase-active mutations were generated. The pEGFP-N3 plasmid was purchased from Clontech (Mountain View, CA) as plasmid transfection control.

Luciferase assays

The human CBF β promoter in pLightSwitch_Prom plasmid (SwitchGear Genomics, Carlsbad, CA) encodes the wild type promoter sequence (1 kb upstream of the CBF β gene 5'-UTR). CDK11^{P110}, CDK11^{P58}, and CDK11^{P110} mutant plasmids were transfected with Lipofectamine™ 3000 (Invitrogen, Grand Island, NY). LightSwitch™ Promoter Reporter Vector (SwitchGear Genomics) was transfected as an internal transfection control. Cells were collected 48

h after transfection and lysates were prepared for luciferase assays following the manufacturers' protocols (Switch-Gear Genomics). Luciferase activity with LightSwitch Assay Reagents was measured by the Perkin Elmer Victor 3 Microplate Readers (Waltham, MA).

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed with the U-2OS and KHOS cell lines according to the manual from Cell Signaling Technology (MA). The CBF β promoters were amplified using PCR. In brief, 2×10^7 cells were transfected with CDK11 siRNA. Forty-eight hours after transfection, cells were crosslinked in 1% formaldehyde for 10 min at room temperature. Crosslinking was terminated by incubation of cells with glycine solution for 5 min. Cells were washed and chromatin was sonicated to a length of 100–900 bp using a VIRSONIC 100 Ultrasonic Homogenizer (Woburn, MA), while samples were maintained on ice at 4 °C. Protein G Magnetic beads (SimpleChIP® Enzymatic Chromatin IP Kit (Magnetic Beads), Cell Signaling Technology, Danvers, MA) were washed with blocking solution and pre-coated overnight with anti-CDK11 antibody (sc-928, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Histone H3 (SimpleChIP®Kit, Cell Signaling Technology) as a positive control, or IgG as a negative control. IP samples were incubated overnight at 4 °C with rotation. Beads were collected by magnetic attraction using a magnetic rack. After washing, protein-DNA complexes were eluted and crosslinks were reversed by incubating the suspensions for 2 h at 65 °C. DNA was purified using spin columns. ChIP DNA was analyzed with PCR using positive and negative controls. The ChIP signal was normalized to the input sample and total DNA content. ChIP PCR primers were designed in the proximal regions of CBF β gene promoters, around genomic locations where potential CDK11-binding elements were located. Standard PCR method was performed following the manufacturer's ChIP Protocol (Cell Signaling Technology). PCR products were confirmed by sequencing and compared with CBF β promoters.

Western blotting assay

Total protein lysates from the osteosarcoma cells were harvested by $1 \times$ RIPA Lysis Buffer (Upstate Biotechnology, Charlottesville, VA) plus complete protease inhibitor cocktail tablets (Roche Applied Science, IN, USA). Protein Assay Reagents (Bio-Rad, CA) and a SPECTRAmax Microplate Spectrophotometer from Molecular Devices (Sunnyvale, CA) were applied to evaluate the protein concentrations. The primary antibodies for CDK11 (1:1000 dilution) and β -actin (1:2000 dilution) were purchased from Santa Cruz and Sigma-Aldrich, respectively. Secondary antibodies IRDye® 800CW or IRDye® 680LT were purchased from LI-COR (Biosciences, NE). Western blot analysis was performed as previously

reported (Duan et al., 2008). Membrane signals were detected using the Odyssey infrared imaging system. Quantification analysis of western blot values was performed with Odyssey software 3.0 (LI-COR Bioscience, NE).

MTT cell proliferation assay

KHOS or U-2OS cell lines were transfected with CBF β siRNA or non-specific siRNA. 3×10^3 cells were seeded into each well of 96-well plates. Complete growth medium without antibiotics was added into each well to a volume of 100 μ l in triplicate. After 72 h of incubation, 20 μ l of MTT (5 mg/mL, dissolved in PBS, Sigma-Aldrich) was added to each well and the cells were cultured for 4 h at 37 °C. The MTT formazan product was dissolved with acid isopropanol. The absorbance at a wavelength of 490 nm (A490) was measured on a SPECTRAmax Microplate Spectrophotometer from Molecular Devices (Sunnyvale, CA). All results were analyzed by GraphPad Prism 5 software (San Diego, CA).

Wound healing migration assay

Migration activity was detected by multiple scratch wounds assay. In brief, 1×10^5 cells per well were plated into 12-well plates and transfected with CBF β siRNA. Three parallel lines were made in confluent cell cultures with a 200 μ l tip. After suspended cells were washed away with serum-free medium, the cultures were fed with complete medium again. The wounds were observed at 0, 8, and 24 h after wounding, and photographed via microscope (Nikon Instruments, Inc.). Three images were taken per well at each time point using a 10 \times objective, and the distance between the two edges of the scratch (wound width) was measured at 10 sites in each image. The cell migration distance was defined as the wound width at the 0 h time point minus the wound width at each time point and then divided by two.

Matrigel invasion assay

The matrigel invasion assay was performed with the BD BioCoat™ Matrigel™ Invasion Chamber (Becton-Dickinson, MA) following the manufacturer's instructions. CBF β siRNA or non-specific siRNA transfected cells (5×10^4 cell/plate) were seeded into the upper chamber of each well in serum-free medium, and complete medium was put into the bottom chamber. The invasion chamber was incubated for 24 h at 37 °C under 5% CO₂. Afterward, non-invading cells were removed by scrubbing from the upper surface of the membrane with cotton-tipped swabs. After washing the cells with medium, complete medium with 1 μ g/mL Hoechst 33342 (Invitrogen) was used to stain nuclei of invading cells for 5 min. Images were acquired by a Nikon Eclipse Ti-U fluorescence microscope and phase contrast microscope equipped with a SPOT RT digital camera. The number of invading cells was counted in three images per membrane by microscopy using a 20 \times objective.

Immunohistochemical staining on TMA

The expression levels of CBF β and CDK11 were determined based on the immunohistochemistry protocol (Paraffin) from Cell Signaling Technology (Beverly, MA) as previously described (Zhang et al., 2013). Briefly, 5- μ m-thick array sections were baked at 60 °C for 1 h, dewaxed with xylene (three times for 5 min each), transferred through 100% ethanol (twice for 5 min each), rehydrated through graded alcohol, and then immersed in deionized water for 10 min. Antigen retrieval was processed with Target Retrieval Solution (Dako, North America, Inc., CA). Afterward, the slide was washed with PBS twice for 5 min. Following the process of antigen retrieval, endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide. Then, blocking solution (Cell Signaling Technology) was applied for 1 h at room temperature, and primary CBF β or CDK11 antibody (1:50 dilution, in 1% bovine serum albumin PBS) was probed at 4 °C overnight in a humidified chamber. Each step was accompanied by three Tris-buffered saline (TBS) rinses, and the bound antibody on the array was detected by using SignalStain[®] Boost Detection Reagent (Cell Signaling Technology) and SignalStain[®] DAB (Cell Signaling Technology). Finally, sections were counterstained with Hematoxylin QS (Vector Laboratories) and the slide was mounted with VectaMount AQ (Vector Laboratories) for long-term preservation. CBF β and CDK11 expressions were evaluated according to visible nuclear staining and calculated by the percentage of positive cells among the entire spot. Thereby, CBF β staining patterns were categorized into 6 groups: 0, no nuclear staining; A, 1+, < 10% of cells stained positive; B, 2+, 10 to 25% positive cells; C, 3+, 26 to 50% positive cells; D, 4+, 51 to 75% positive cells; and E, 5+, > 75% positive cells. CBF β and CDK11 staining images were obtained using a Nikon Eclipse microscope (Nikon Corp) with a SPOT RT digital camera (Diagnostic Instruments Inc.).

Results

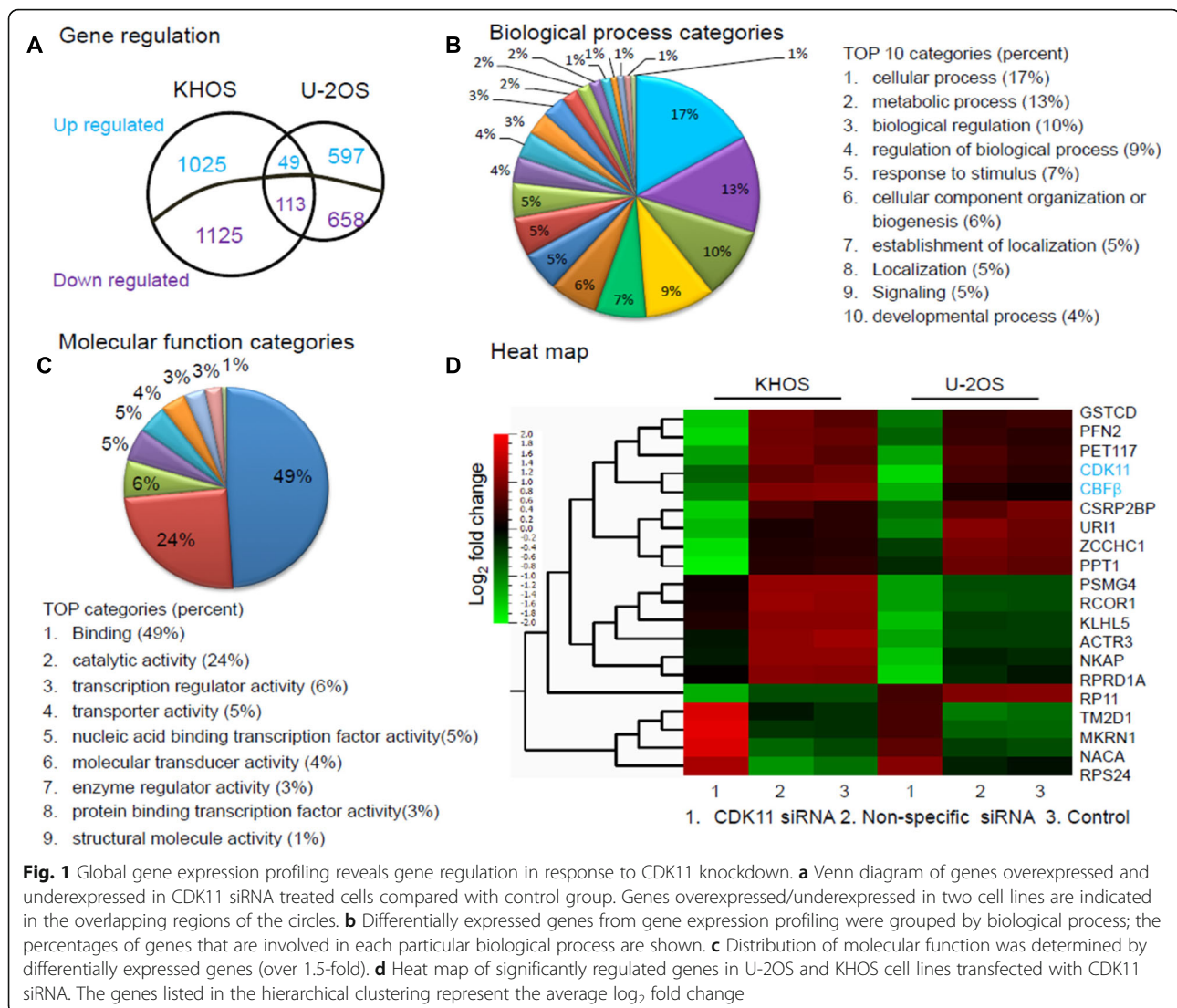
Expression profiling of gene regulation by CDK11

To identify the genes regulated in response to knockdown of CDK11 expression, KHOS and U-2OS osteosarcoma cell lines were transfected with CDK11 siRNA and their gene expression profiles were analyzed by Genesifter. The microarray data from this study has been submitted to the database of Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) and assigned the GEO accession number GSE73422 [26, 27]. Genome-wide gene profiling identified 162 differentially expressed genes (≥ 1.5 log fold alteration in expression, $P < 0.05$) in both KHOS and U-2OS cell lines with CDK11 siRNA knockdown (Fig. 1a and Additional file 2: Table S1), as compared with non-specific siRNA treated cells or untreated cells. Analysis by biological category revealed

that these differentially expressed genes are enriched in cellular processes (17%), metabolic processes (13%), and biological regulation (10%) (Fig. 1b). Further analyses were focused on the molecular function of these differentially expressed genes. We observed that the genes involved in protein or DNA binding ($n = 74$), enzyme catalytic activity ($n = 37$), and transcription regulator activity ($n = 9$) comprised the most common molecular function categories (Fig. 1c). This indicated that CDK11 is involved in extensive biological regulation and plays important roles in transcription regulation. Further analysis of the altered gene clusters using Qlucore software revealed the top 20 significantly differentially expressed genes, which are shown in a heat map as Fig. 1d and Additional file 3: Table S2 ($P < 0.05$). Among these genes, 16 were downregulated, whereas the remaining genes were upregulated. CBF β was the most highly correlated expressing gene with CDK11.

CBF β expression depends on CDK11 expression on transcriptional and translational levels

The relationship between CDK11 and CBF β was evaluated in KHOS and U-2OS cell lines using quantitative RT-PCR. We observed knockdown of CDK11 significantly decreased transcript levels four-fold and five-fold in KHOS and U-2OS cell lines (Fig. 2a). In accordance with decreasing CDK11 mRNA, there was a three-fold decrease in CBF β mRNA expression compared with controls in both KHOS and U-2OS cell lines (Fig. 2a). To investigate whether the downregulation in expression observed at the mRNA level was also reflected at the protein level, cells were transfected with increasing doses of CDK11 siRNA. CDK11 protein expression was suppressed by CDK11 siRNA in a dose-dependent manner, while CBF β protein was also reduced in a dose-dependent manner (Fig. 2b). These results demonstrated that CBF β expression depends on CDK11 expression at the translational level. In normal human cells, the expression of CDK11 is tightly regulated; for example, in the normal human osteoblast cell lines HOBc and NHOst, there are extremely low or almost undetectable levels of CDK11, while CBF β levels are correspondingly low in normal human osteoblast cell lines and exhibit high expression in osteosarcoma cell lines (Fig. 2c). MNNH/HOS and 143B are osteosarcoma cell lines that are highly tumorigenic and are able to induce pulmonary metastases in xenograft mouse models in vivo [28, 29]. Interestingly, CDK11 expressions in MNNH/HOS and 143B cell lines were higher than in U-2OS and KHOS cell lines. Consistent with this, the protein expressions of CBF β were also higher in the MNNH/HOS and 143B cell lines than the non-metastatic osteosarcoma U-2OS and KHOS cell lines (Fig. 2d). Four out of eight osteosarcoma tissue samples showed that both CBF β and CDK11 proteins were highly expressed (Fig. 2E).

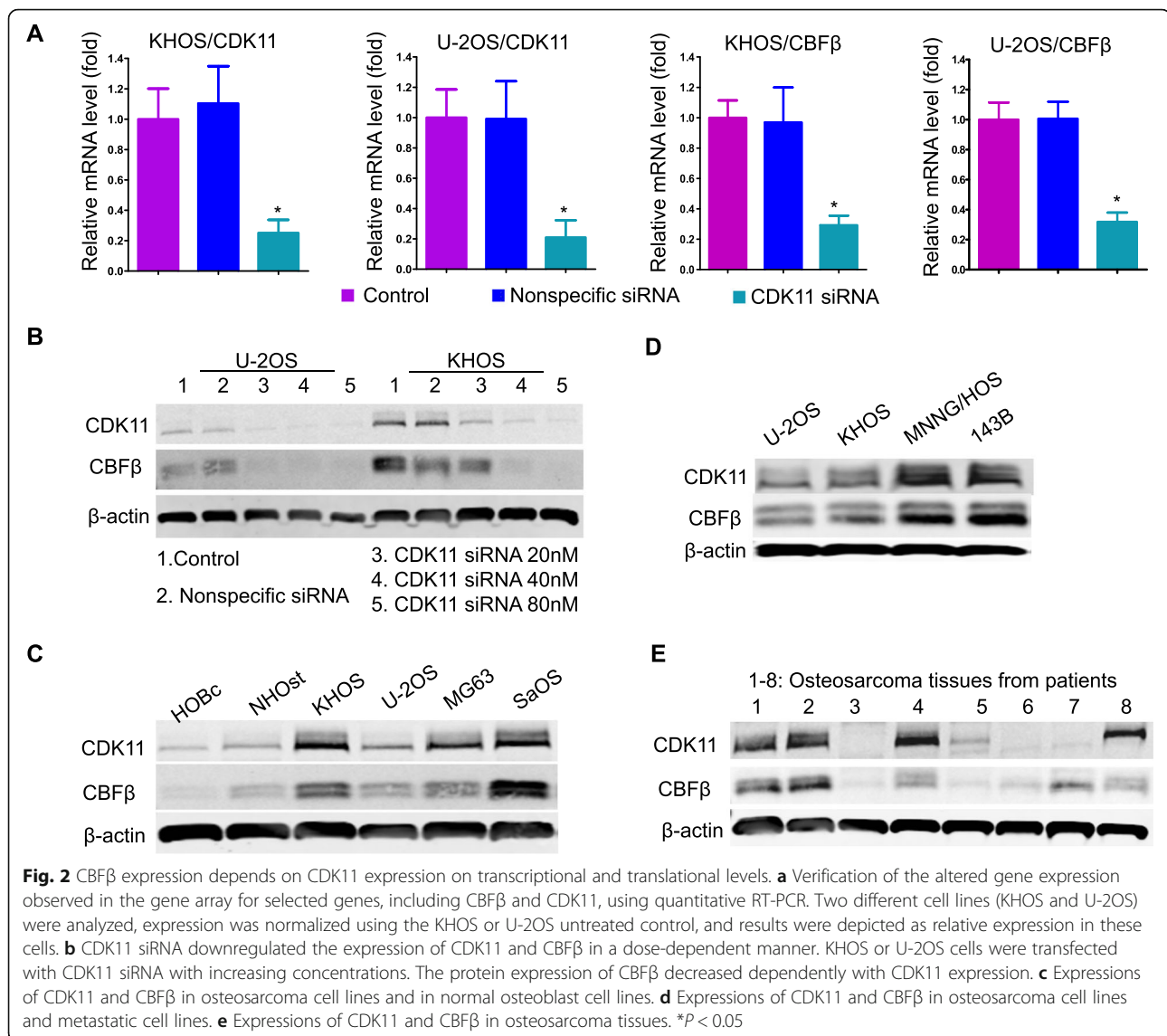


CDK11^{P110} localizes to the nucleus and is required for the transcriptional regulation of CBFβ genes

The human CDK11 gene is located on chromosome 1p36.33 and contains 20 exons and 19 introns (Fig. 3a). To determine whether CDK11^{P58}, CDK11^{P110}, or both isoforms are associated with transcription, we first examined the subcellular localization of these two isoforms. Transfection with different CDK11 isoforms fused with GFP expression vectors revealed that CDK11^{P110} was localized in the nucleus of three different osteosarcoma cell lines, while CDK11^{P58} was mainly localized in the cytoplasm (Fig. 3b). We then generated a U-2OS cell line stably expressing the GFP-CDK11^{P58} or the GFP-CDK11^{P110} protein (Fig. 3c). Consistent with transient transfection, the selected U-2OS- GFP-CDK11^{P110} cell line showed nuclear localization (Fig. 3c). Also as expected, the selected U-2OS-GFP-CDK11^{P58} cell line localized largely in the cytoplasm. Moreover, CDK11^{P110}

was highly and constantly expressed in U-2OS and KHOS cell lines, whereas CDK11^{P58} was almost undetectable in these cells (Fig. 3d). Previous studies have shown that CDK11^{P110} protein is mainly localized in the nucleus of osteosarcoma cells both in cell lines and in tumor tissues [13]. Similar cellular localization of CDK11^{P110} has been demonstrated in other types of cancer cells, including in breast cancer and ovarian cancer [8, 9, 14]. Because CDK11^{P110} has been found broadly distributed onto chromatin and is involved in some aspects of transcription [20, 22], we investigated whether CBFβ was regulated by CDK11^{P110} or by CDK11^{P58}.

We measured the regulatory activity of CDK11 on the CBFβ promoter using luciferase reporter assays. The length of the CBFβ promoter region was 1062 nucleic acid base pairs (bp) from -760 to +302 relative to the transcriptional start site. To study how CDK11 regulates CBFβ expression, a DNA fragment containing this region was

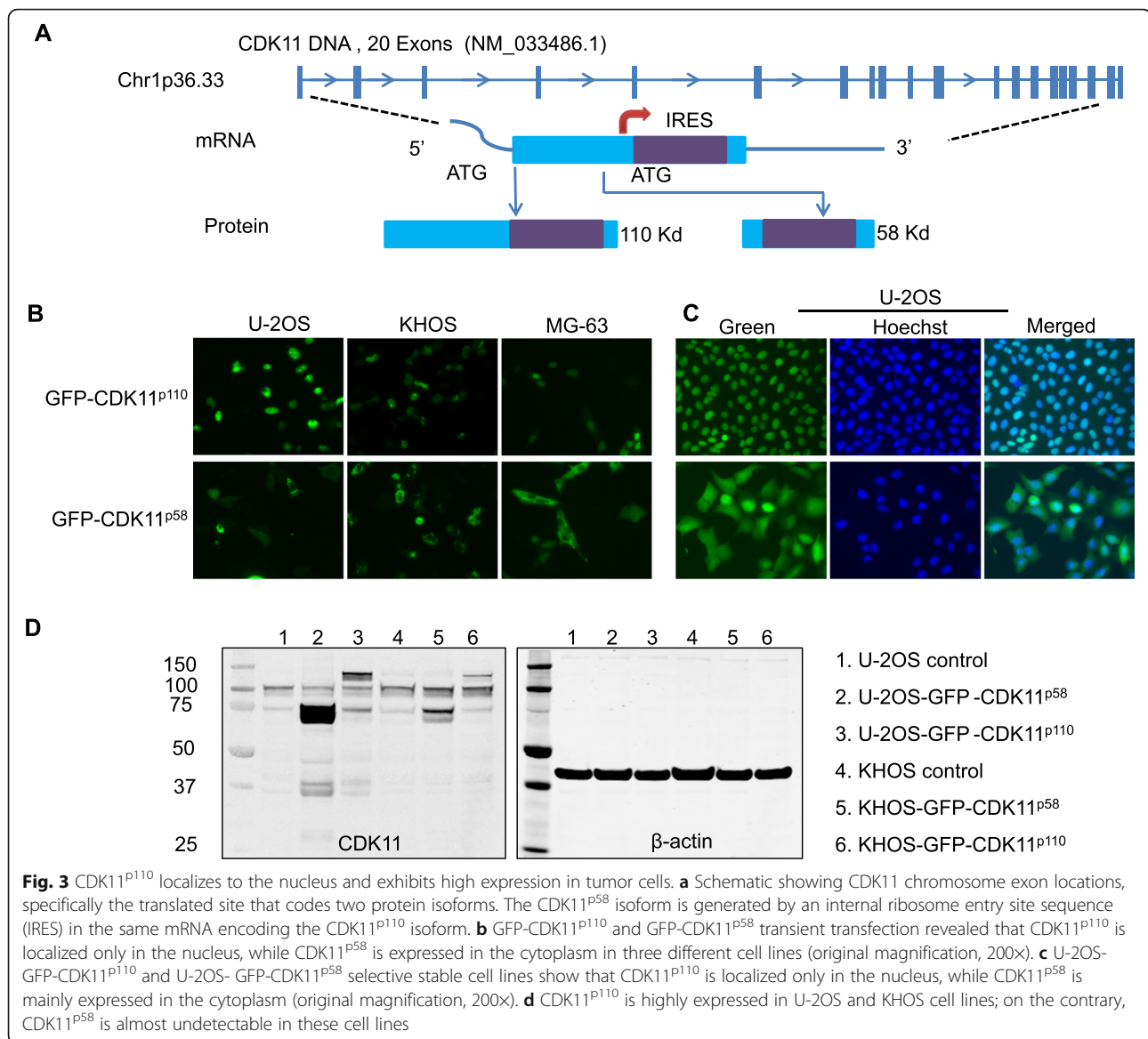


inserted upstream of the RenSP luciferase gene in the LightSwitch Promoter Reporter vector; once protein is bound to the CBFβ promoter region, it activates or enhances the luciferase activity (Fig. 4a). Transient transfection assays were conducted in KHOS and U-2OS cell lines. Co-expression of the CBFβ promoter luciferase vector with CDK11^{P110} increased luciferase activity seven-fold and six-fold in U-2OS and KHOS cells, respectively (Fig. 4b and c). Furthermore, CDK11^{P110} induced CBFβ promoter activity in a dose-dependent manner in both of the osteosarcoma cell lines. These results suggest that CDK11^{P110} acts as a transcriptional activator on the CBFβ promoter in these cell lines. We then investigated whether CDK11^{P58} was also involved in CBFβ regulation. As shown in Fig. 4d, in the presence of the CDK11^{P110} expression plasmid, CBFβ promoter

activity was dramatically increased. However, the CDK11^{P58} expression vector had almost no effect on CBFβ promoter activity in the KHOS cell line. Similar to the KHOS cell line, CDK11^{P58} expression also showed no significant effect on CBFβ promoter activity in the U-2OS cell line (Fig. 4e). These data imply that CDK11^{P110}, but not CDK11^{P58}, increases CBFβ transcriptional activity.

CDK11^{P110} directly upregulates CBFβ expression by binding to the CBFβ promoter

DNA-protein interactions are crucial to fundamental cellular events, including gene transcription. Thus, these studies hold the key to our understanding of mechanisms underlying deregulated gene expression in human diseases, such as in cancer. The luciferase assay showed



that CDK11^{p110} regulates the CBF β promoter. However, whether this regulation was induced by CDK11^{p110} directly binding to the CBF β promoter or not remains unclear. In order to identify the direct interaction between CDK11^{p110} and CBF β , the chromatin immunoprecipitation (ChIP) assay was applied. The ChIP assay is a powerful method to study interactions between proteins and a specific genomic DNA region (protein-DNA interaction). Recruitment of proteins to promoters of specific genes is a common mechanism to activate or enhance gene transcription [30]. In the ChIP assay, the level of enrichment of the DNA sequence is determined relative to the total amount of input DNA (2% of input) (Additional file 1: Figure S1). To determine which site(s) might bind with the CDK11^{p110} protein, we designed four pairs

of primers whose corresponding regions almost cover the whole length of the CBF β promoter (– 680 to 287 bp) (Fig. 5a). Of the four pairs of primers, significant enhancement of CDK11^{p110} binding was observed by ChIP in Primer 3 (– 436/– 227) and Primer 4 (– 680/– 547) (Fig. 5a). Consistently, knock-down of CDK11^{p110} by siRNA significantly decreased the binding efficiency of the CBF β promoter region with Primer 3 (Fig. 5b). Sequencing of Primer 3 ChIP products confirmed that binding area located at the CBF β promoter area in the genome (data were not shown). These data support an important role for CDK11^{p110} in mediating CBF β expression in osteosarcoma through its ability to directly bind to the two unique sites of DNA sequences in the CBF β promoter.

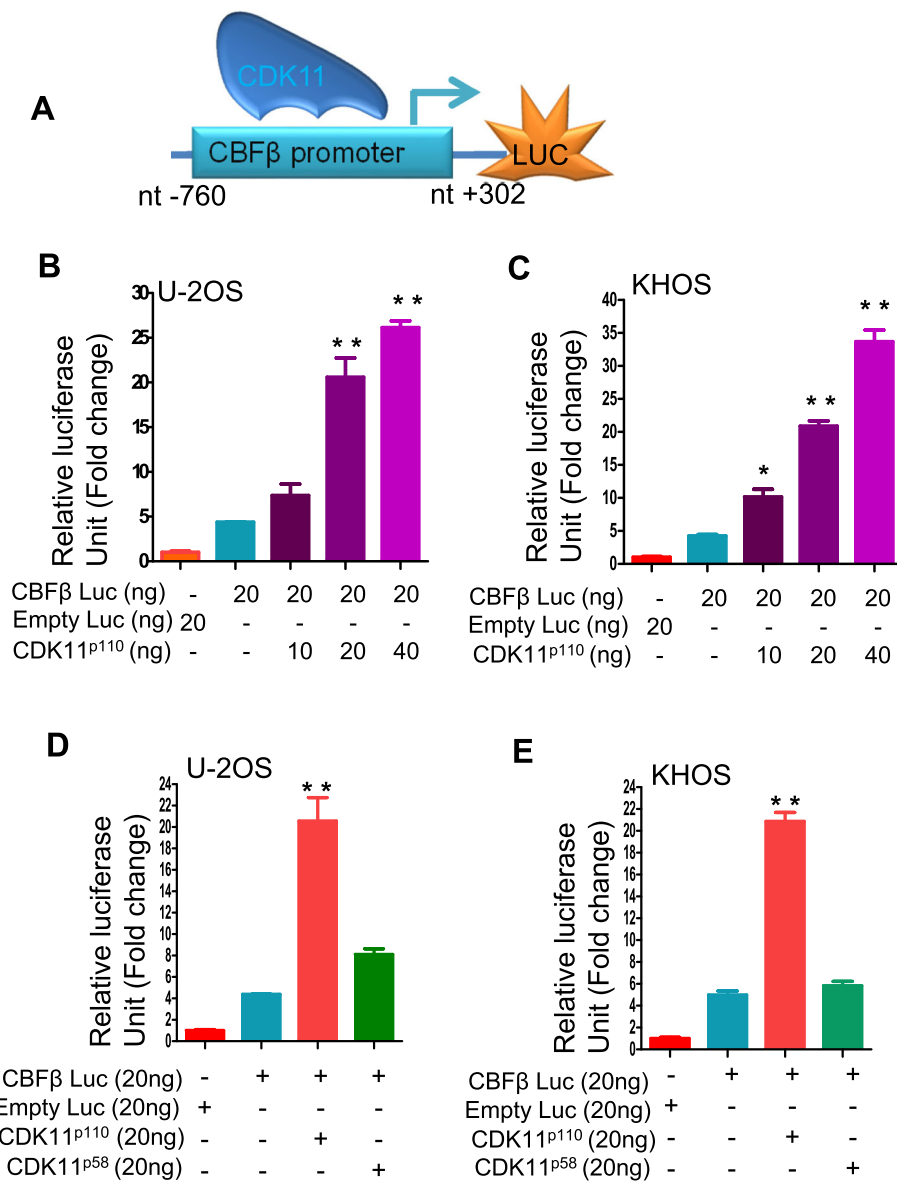
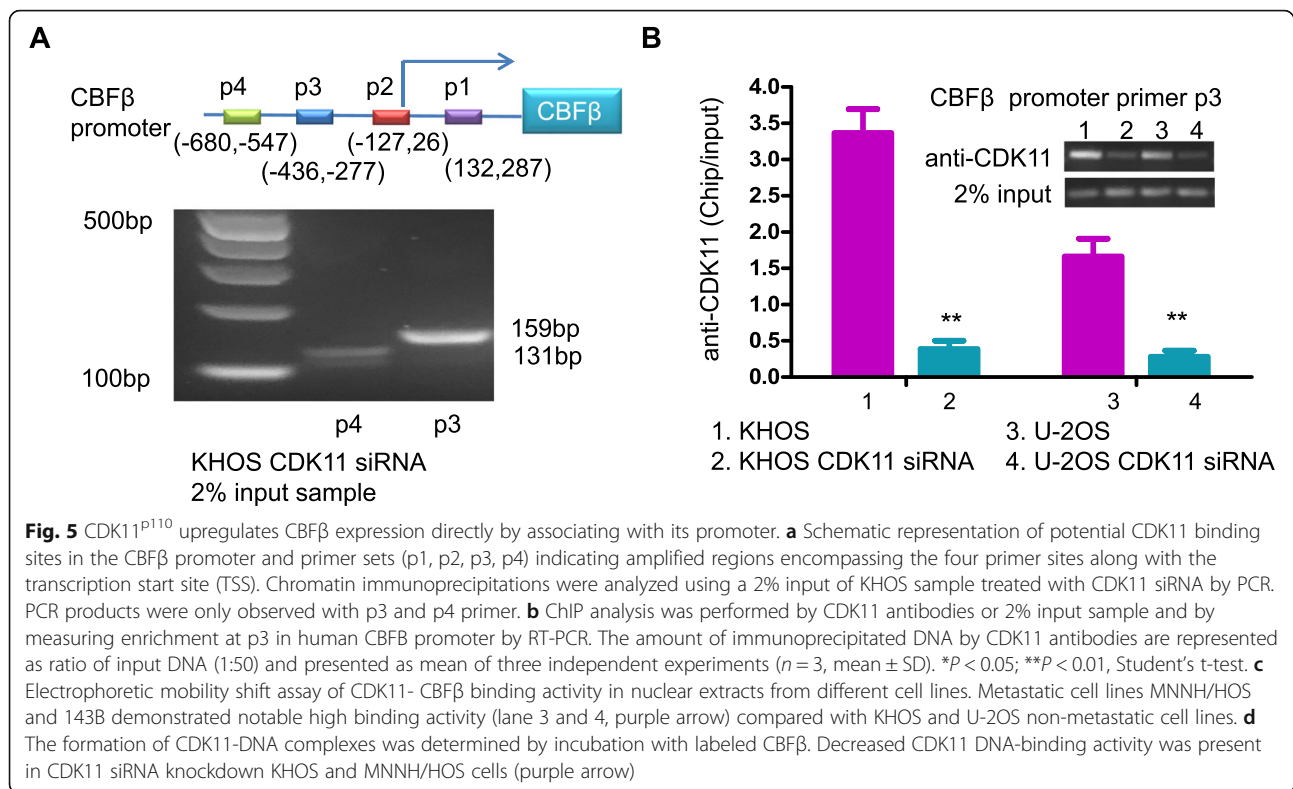


Fig. 4 CDK11^{p110}, not CDK11^{p58}, regulates promoter activity of CBFβ genes. **a** Schematic representation of the promoter-luciferase construct showing the CBFβ promoter region. **b** CDK11^{p110} upregulates CBFβ promoter-luciferase in a dose-dependent manner in the U-2OS cell line. U-2OS cells were transfected with the CBFβ promoter-luciferase construct. Co-expression was conducted with an empty expression vector (control) or CDK11 expression vectors. Error bars indicate standard deviation and are from at least three replicates. **c** CDK11^{p110} upregulates CBFβ promoter-luciferase in a dose-dependent manner in the KHOS cell line. KHOS cells were transfected with the CBFβ promoter-luciferase construct. Co-expression was conducted with an empty expression vector (control) or CDK11 expression vectors. Error bars indicate standard deviation and are from at least three replicates. **d** CDK11^{p110}, not CDK11^{p58}, activates the CBFβ promoter-luciferase in the KHOS cell line. **e** CDK11^{p110} significantly increases CBFβ promoter-luciferase in KHOS cell line. **f** Schematic showing major structural features of the CDK11^{p110} protein. CDK11^{p110} kinase-dead or kinase-active mutations were generated. The asterisk represents the amino acid that was mutated to create kinase-dead or active mutations. **g** In the U-2OS cell line, the C-terminal kinase domain mutation of CDK11^{p110} failed to affect CDK11^{p110}-mediated CBFβ activation. **h** The C-terminal kinase domain mutation of CDK11^{p110} also did not change CBFβ promoter activity in the KHOS cell line. **P* < 0.05, ***P* < 0.01

CBFβ contributes to the proliferation and metastatic behavior of osteosarcoma cells

CDK11 plays a crucial role in cancer cell proliferation and growth, including in osteosarcoma. The current study has

found that CDK11 protein can directly regulate CBFβ expression. Although CBFβ has been reported to play important roles during skeletal development in previous studies, the role of CBFβ in osteosarcoma remains

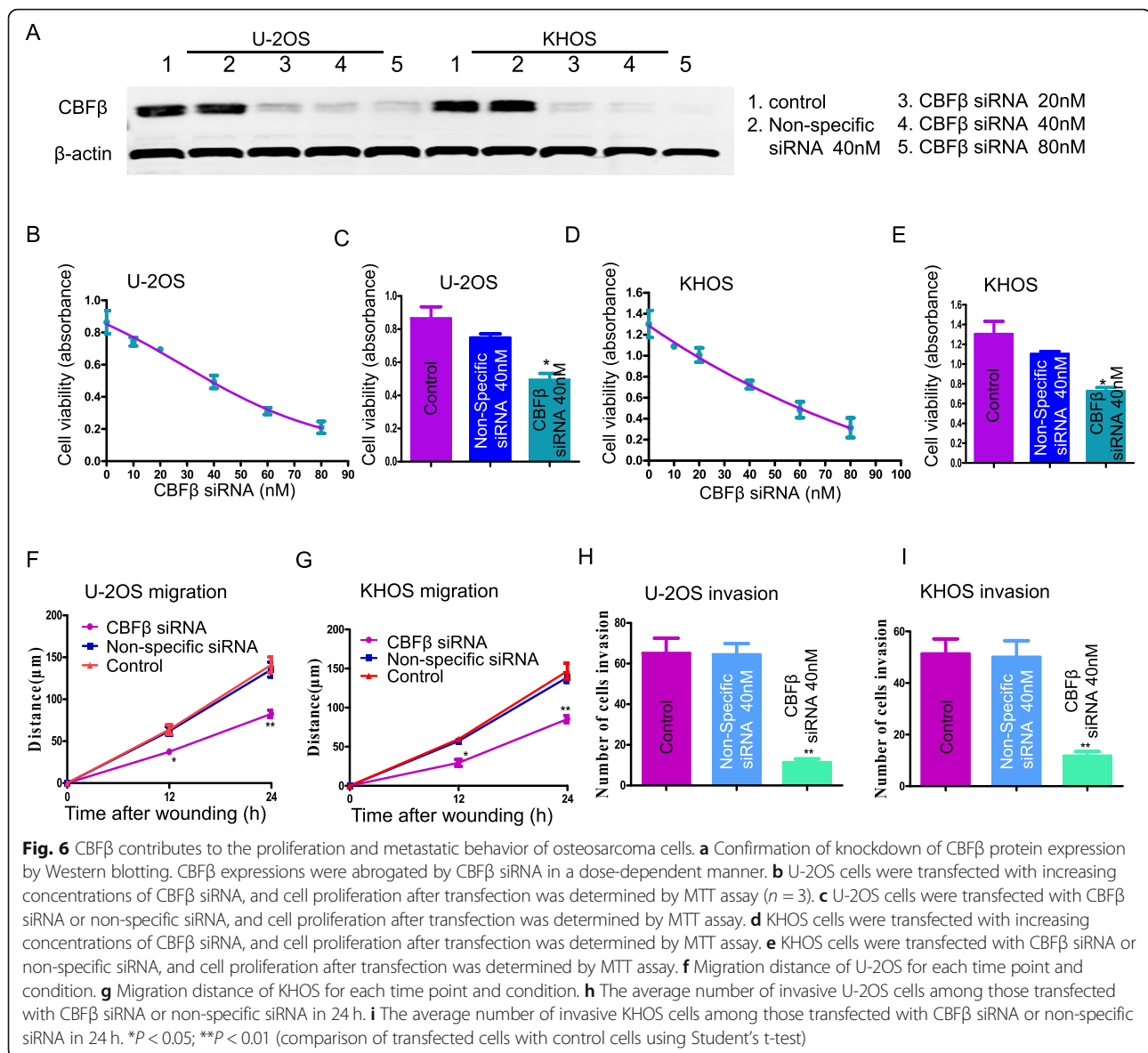


unknown. Therefore, we further investigated the functional role of CBFβ in osteosarcoma. Western blotting of cells with CBFβ siRNA demonstrated that CBFβ expression can be inhibited in a dose-dependent manner (Fig. 6a). Depletion of CBFβ by siRNA resulted in dose-dependent cell growth inhibition in both KHOS and U-2OS cell lines, which was not observed with non-specific siRNA transfection (Fig. 6b to e). Since CBFβ showed higher expression in metastasis cell lines MNNH/HOS and 143B as compared with non-metastatic cell lines U-2OS and KHOS, we examined whether CBFβ knockdown by siRNA transfection could affect the migratory and invasive activities of osteosarcoma cells by wound healing assay and Matrigel invasion assay. The migration assay showed that downregulation of CBFβ significantly suppressed the migration of U-2OS and KHOS cells (Fig. 6f and g). Consistently, Matrigel invasion assays identified that the average numbers of osteosarcoma cells that were invading through the Matrigel after treatment with CBFβ siRNA were significantly lower than the blank control and the non-specific siRNA groups (Fig. 6h and i). Taken together, these results demonstrate that CBFβ plays a role in promoting osteosarcoma cell growth and invasion, similar to the roles of CDK11 in cancers [9, 11, 13, 15].

High CBFβ expression correlates with CDK11 expression and contributes to reduced overall survival in osteosarcoma

CBFβ has been shown to contribute to malignancy in several human solid cancer types [31–34]. We further

examined whether the levels of CDK11 correlated with the level of CBFβ in tumor cells by using a high-density tissue microarray (TMA), which included tissues from 73 available characteristics information of osteosarcoma specimens (Additional file 4: Table S3) [35]. Immunohistochemical analysis showed that CBFβ and CDK11 expression was quite variable in these samples. Among the evaluable specimens, 63.3% of CDK11 positive staining specimens were classified as CDK11-high patients (63.3%), and 65.6% of CBFβ positive staining specimens were categorized as CBFβ -high patients (Fig. 7a). These results indicated that the levels of CDK11 and CBFβ were highly correlated (correlation coefficient $R^2 = 0.7729$, $P < 0.0001$, Fig. 7b). Kaplan-Meier analysis showed that osteosarcoma patients had a lower overall survival rate in the high CDK11 expression group compared with patients in the low CDK11 expression group ($P < 0.0001$) (Fig. 7c). Similarly, higher expression of CBFβ in osteosarcoma patients also predicted a significantly reduced survival rate ($P = 0.0050$) (Fig. 7d). Most importantly, the CDK11 expressions in 12 metastatic patients were dramatically higher in their metastatic samples than in their primary specimens ($P = 0.0003$) (Fig. 7e). Likewise, the CBFβ expressions in the samples of the metastatic patients were significantly higher than in their matched primary specimens ($P = 0.0028$) (Fig. 7f). The results above, coupled with the correlation of high CBFβ levels with metastatic behavior in breast, prostate, and ovarian cancer



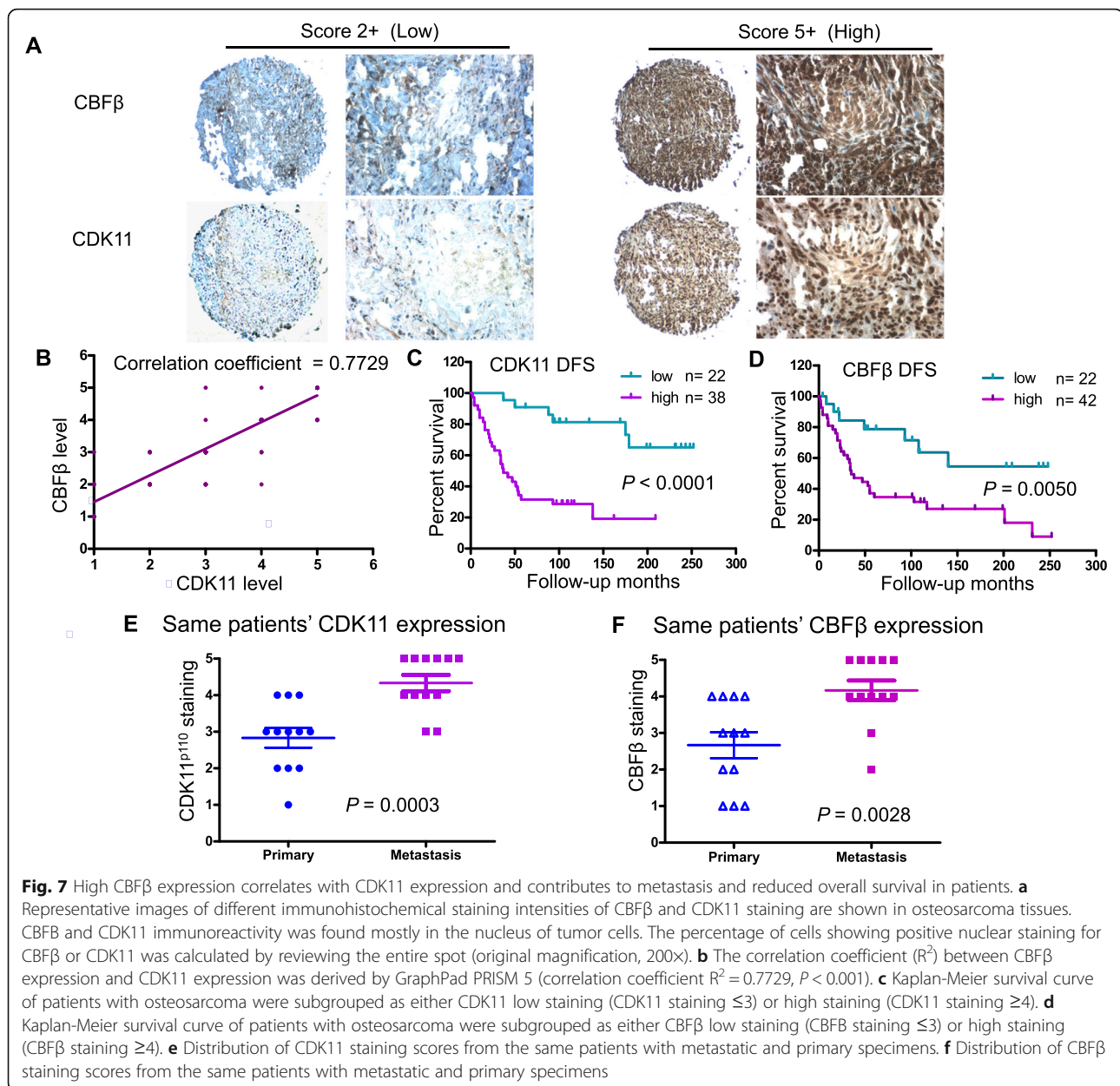
cells, support the notion that high CBF β expression in the primary tumor may contribute to metastatic behavior.

Discussion

In the current study, we have established that CBF β is a CDK11 transcriptional target and promotes osteosarcoma cell growth and metastasis. We utilized global gene expression profiles after CDK11 knockdown by siRNA and discovered that CDK11 increases CBF β -dependent transcriptional activation in osteosarcoma cells. Several protein DNA interaction experiments confirmed CBF β as a direct transcriptional target of CDK11^{P110}, but not CDK11^{P58}. High expression of CBF β was shown to be associated with poor outcome in osteosarcoma patients. This is the first report demonstrating a transcriptional regulation of CBF β by CDK11 and that

CBF β can be a potential therapeutic target for osteosarcoma treatment.

Although CDK11 siRNA inhibits widespread transcription of a number of genes, CBF β was the most significantly downregulated gene in the analysis. CBF β , also known as Polyomavirus Enhancer-Binding Protein 2 Beta Subunit (PEBP2B), can positively regulate transcription from the RNA polymerase II promoter. CBF β plays an indispensable role in skeletal development and homeostasis in various skeletal cell types [36–38]. Moreover, CBF β is essential for invasion and xenograft tumor growth in some solid tumors, including in breast, prostate, and ovarian cancers [31, 32, 39, 40]. Therefore, further validations on the correlation between CDK11 and CBF β expression were performed. There are at least three tiers of evidence that demonstrate that CDK11



mediates CBF β pathway transcriptional regulation. Firstly, genome-wide gene profiling identified that CDK11 regulates CBF β on a transcriptional level and that CBF β protein expression depends on CDK11 protein expression. Secondly, promoter-luciferase assays demonstrated that CBF β is regulated by CDK11^{P110}, which is located only in the nucleus, while CDK11^{P58} is mainly expressed in the cytoplasm. Despite data clearly indicating that CDK11^{P110} interacts with transcriptional proteins and that it is required for high level in vitro transcription, the exact role of CDK11^{P110} in transcription in vivo and the biological substrates of the kinase remain undetermined [41]. Our results demonstrated that CDK11^{P110} induced CBF β promoter activity in a dose-dependent manner in two

osteosarcoma cell lines. Interestingly, we observed that CDK11^{P110}, but not CDK11^{P58}, increased CBF β transcriptional activity. Consistent with this, CBF β expression is not cell cycle-regulated and is maintained at high levels during the cell cycle in osteosarcoma cells [42]. Similarly, CDK11^{P110} expression is ubiquitous and constant throughout the cell cycle, while CDK11^{P58} is only specifically and transiently expressed during G2-M phase of the cell cycle. The structural features of CDK11^{P110} include its DNA binding area located on the N-terminus [21, 41, 43, 44]. BindN, a web-based tool for efficient prediction of DNA binding sites in amino acid sequences, predicted that 73.3% of DNA-binding residues are located on the N-terminus of

CDK11^{p110} [45]. Thirdly, the ChIP assay confirmed that CBF β is a direct transcriptional target of CDK11. Increasing evidence has revealed that the CDK family specifically occupies the promoters of a subset of genes [46–49]. In one study, ChIP confirmed CDK8 occupancy on the MYC promoter containing β -catenin/TCF elements [50]. Moreover, ChIP-seq demonstrated that CDK7 amplified MYCN mRNA and protein levels by occupying its promoter [51, 52]. Interestingly, CDK11^{p110} has been found broadly distributed on chromatin [22]. In addition, CDK11^{p110} has been proven to participate in transcription regulation initiated at an adenosine deaminase (GC)-rich promoter, while the CBF β promoter region contains high GC content [20]. In this study, for the first time, we identified CBF β as a direct CDK11 target gene and demonstrated that CDK11 binds to the proximal CBF β promoter by ChIP assay. These data collectively verified that CBF β is a sequence-specific target of CDK11^{p110} in human osteosarcoma cells. In the future, it will be of interest to determine whether this model applies to regulation by CDK11 in other systems.

In addition to demonstrating that CBF β is a transcriptional target of CDK11, CBF β showed similar biological functions to CDK11 in osteosarcoma. CDK11 is essential for cancer cell growth and invasion [9, 11, 13–16, 34]. In accordance with previous studies, we demonstrated that CBF β is highly expressed in osteosarcoma cell lines, and especially highly expressed in osteosarcoma metastasis cell lines. We subsequently demonstrated that CBF β depletion reduces cell viability, and migration and invasion activities of metastatic osteosarcoma cells. CDK11^{p110} expression is ubiquitous and constant throughout the cell cycle, and CBF β is maintained at high levels during the cell cycle in osteosarcoma cells [42]. Aberrant expression of CBF β proteins has been linked to pathological events in cancer cells [31, 32, 39]. Expression of CBF β is essential for cancer cell invasion and migration, indicating that CBF β may have an oncogenic function in tumor etiology [32, 39, 53–55]. Notably, expression levels of CDK11 and CBF β protein are both extremely low in normal bone osteoblast cells, but are significantly higher in osteosarcoma cell lines. These may be explained by the fact that CDK11 and CBF β possess similar essential roles in development. CDK11 knockout mice display an earlier lethality during the blastocyst stage of embryonic development [56]. Deletion of the CBF β subunit revealed impaired bone formation and caused embryonic lethality [57].

CBF β is the most frequently mutated and rearranged gene in human leukemias and plays an important role in hematologic malignancies [33, 55, 58]. Although no genetic defects have been reported for CBF β in other types of human cancers, such as in osteosarcoma, there is a report of multiple CBF β gene copies detected by FISH in a single case of granulocytic sarcoma associated with

myeloid leukemia [59]. In line with previous gene profiling, high expression of CBF β mRNA has been found in KHOS and U-2OS cell lines, and the expression of CBF β is CDK11 expression dependent. Consistent with the correlation of CBF β and CDK11 on the mRNA level, high CBF β protein expression correlates with CDK11 expression and contributes to diminished overall survival. Furthermore, CDK11 and CBF β expressions in metastatic patients were significantly higher than those in their matched primary specimens, which resulted in lower overall survival. Although CBF β has been shown to play crucial roles in leukemia, the correlation between CBF β and clinicopathological features has not been extensively analyzed in solid tumors. The precise role of CBF β has not been fully elucidated, largely due to the complexity of the transcription factor itself. Since amplification and overexpression are generally considered hallmarks of an oncogene, CDK11 mediating CBF β may be oncogenic.

Conclusions

In conclusion, we have taken a genetic approach to understanding CDK11 regulated genes in osteosarcoma. A systematic combination of global gene expression profiling and DNA-protein assays has led to a wealth of information about how CDK11 and proteins interact in osteosarcoma cells. To our knowledge, no similar large-scale genetic analysis has been carried out on CDK11 or other members of CDK gene family. Our study identified CBF β as a direct transcription target of CDK11^{p110}. Importantly, we have found a new molecular mechanism that promotes osteosarcoma cell growth and provided preclinical evidence for the CDK11^{p110}-mediated CBF β pathway as a molecular marker to predict osteosarcoma metastasis risk. These results provide insights into the use of drugs that target CDK11^{p110}-CBF β for human osteosarcoma therapeutics.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s12964-019-0440-5>.

Additional file 1: Figure S1. Crosslinked chromatin were digested and immunoprecipitated. (A) CDK11 siRNA-treated U-2OS and KHOS cells were formaldehyde-crosslinked and chromatin were prepared and digested by sonication into 1–5 nucleosomes in length (150–900 bp). (B) Chromatin immunoprecipitations were analyzed using the KHOS sample treated with CDK11 siRNA by PCR. PCR products were observed with RPL30 primer in Histone H3 Ab sample (lane 2) and input sample (lane 4), but not in the CDK11 antibody (Ab) sample (lane 1) and normal IgG ChIP sample (lane 3).

Additional file 2: Table S1. Top 162 gene list analyzed by Genesifter.

Additional file 3: Table S2. Top 20 gene list analyzed by Qlucore.

Additional file 4: Table S3. Characteristics of osteosarcoma patients and their tumor samples.

Abbreviations

CBF β : Core-binding factor subunit beta; CDK11: Cyclin-dependent protein kinase 11; ChIP: Chromatin immunoprecipitation; FBS: fetal bovine serum;

GFP: Green fluorescent protein; IHC: Immunohistochemistry; MTT: 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide; siRNA: Small interfering RNA; TMA: Tissue microarray

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Authors' contributions

ZD, YF, and JZ designed the research study and experiments. YF, YL, JZ, and ZD conducted experiments, acquired and analyzed the data. YF, ZD, and JS wrote the manuscript. All authors on the list have read and/or revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The microarray data from this study has been submitted to Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) and assigned the GEO accession number GSE73422.

Ethics approval and consent to participate

The study of tissue microarray was approved by the Partners Human Research Committee (#: 2007P-002464). All patients signed a consent form for their clinical information to be used and published for this research.

Consent for publication

All authors state that they give their full permission for the publication of this study.

Competing interests

The authors declare that they have no competing interests.

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